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14. ABSTRACT Our studies demonstrated that blockade of the complement anaphylatoxin C5a receptor (C5aR) reduced tumor growth in syngeneic and Her2 transgenic mouse models of breast cancer. In both models the therapeutic efficacy of C5aR inhibitor was comparable to the efficacy of Listeria monocytogenes-delivered Her2 vaccine (Lm-LLO-Her2). Importantly, C5aR inhibition synergized with Lm-LLO-Her2 in limiting tumor growth. These therapeutic effects were associated with the enhanced recruitment of tumor-specific CD8+ T cells to tumors. Notably, C5aR inhibition alone contributed to this recruitment and induced tumor-specific T cell responses at the periphery. The induction of the robust anti-tumor T cell responses by various treatments resulted likely from the attenuation of tumor mediated immunosuppression, since we observed that Lm-LLO-Her2, C5aR inhibition and the combination of Lm-LLO-Her2 with C5aR inhibition reduced infiltration of tumors by myeloid-derived suppress cells (MDSCs). The C5aR blockade impacted MDSC infiltration of tumors more than Lm-LLO-Her2. Overall, our data indicate that complement inhibition can become a novel immunotherapy for breast cancer patients in a form of monotherapy or in the combination with other treatment modalities.					
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Introduction

Studies proposed in this application are design to address whether blocking of the complement anaphylatoxin C5a receptor (C5aR): improves the effectiveness of anti-Her2/neu immunization in inducing the regression of primary breast tumors in a transgenic model of breast cancer (Aim 1), reduces the extent of metastatic spread of breast carcinoma and improves the effectiveness of anti-Her2/neu immunization in limiting the growth of breast cancer metastases in a model of spontaneously metastasizing breast cancer (Aim 2). We will also dissect mechanisms by which C5aR blockade improves efficacy of anti-Her2/neu immunization in curing advanced breast carcinoma and its metastases (Aim 3). This research involves transgenic and syngeneic models of breast cancer. Tumor bearing mice will be subjected to treatment with various combinations of C5aR inhibitor and Her2/neu-targeting vaccine. The impact of these treatments on tumor growth will be monitored and various features of anti-tumor immune responses and immunosuppression mechanisms will be evaluated.

Body:

The specific aims of this application are:

Aim 1 Determine whether blocking C5aR improves the effectiveness of anti-Her2/neu immunization in inducing the regression of primary breast tumors. We anticipate achieving this aim within the first year of the project (**months 1-12**).

Aim 2 Determine whether blocking C5aR: (i) reduces the extent of metastatic spread of breast carcinoma and (ii) improves the effectiveness of anti-Her2/neu immunization in limiting the growth of breast cancer metastases. We anticipate achieving this aim in the second year of the project (**months 12-24**).

Aim 3 Dissect mechanisms by which C5aR blockade affects the results of anti-Her2/neu immunization in inducing the regression of or limiting growth of breast carcinoma and its metastases. We anticipate conducting studies in this aim throughout the entire funding period (**months 1-24**).

During this funding period we have conducted several experiments to address **aim 1** and **aim 3** of the original application. The description of results is preceded by the specific aim and task(s) from the statement of work, included in the original application, to which these results pertain.

Aim 1 (TASK 1: months 1-6 and TASK 3: months 6-12)

(i) C5aR blockage synergizes with *Listeria monocytogenes*-based Her2 vaccine (Lm-LLO-Her2) in reducing growth of primary tumors in wild-type FVB/N and Her2/neu transgenic mice.

As proposed in the statement of work, earliest experiments focused on optimizing timing and dosage of vaccine and complement inhibitor in FVB/N wild-type mice. NT-2 or NT-5 cells, which both derived from spontaneous mammary tumors developed in a rat Her2/neu transgenic FVB/N mouse¹, were subcutaneously (s.c.) injected into the rear flanks of FVB/N wild-type mice. Both cell lines were used to assure that the observed effects of treatments are independent, at least to some extent, from a particular tumor model that was used. We found that s.c. injections of 1.0 mg/kg body weight of the hexapeptide inhibitor (C5aR antagonist – C5aRA) were safe and effective in reducing tumor growth and, therefore, this dose was used in all experiments. We used three doses of 10⁷ colony-forming units (c.f.u.) of Lm-LLO-Her2 vaccine

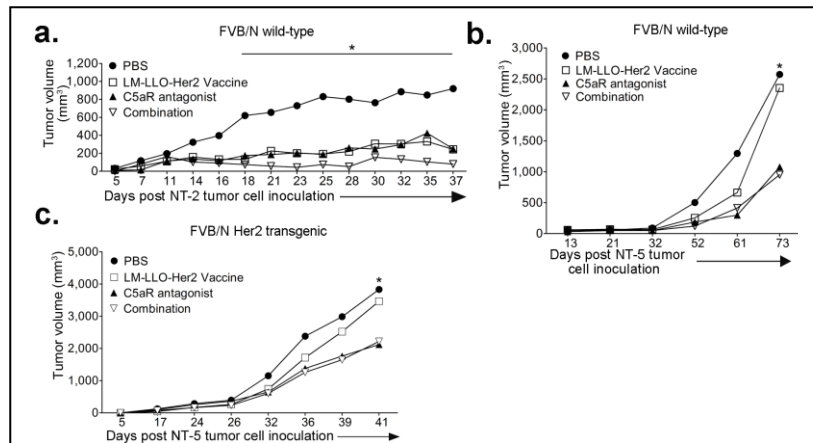


Figure 1 C5a receptor (C5aR) blockage synergizes with *Listeria monocytogenes*-based Her2 vaccine (Lm-LLO-Her2) in reducing growth of primary tumors in FVB/N wild-type and Her-2 transgenic mice. (a-c) Day-to-day tumor volumes of FVB/N wild-type mice inoculated with one million of NT-2 cells (a), one million of NT-5 cells (b), and day-to-day tumor volumes of FVB/N Her2 transgenic mice inoculated with one million of NT-5 cells (c). Mice were administered with Lm-LLO-Her2 or C5aR antagonist or with the combination of Lm-LLO-Her2 and C5aR antagonist (Combination). Control mice received PBS injections only. Data points represent the mean tumor volume for a cohort at respective time points (a-c). (a) Data are representative of one experiment with two control mice and four mice per cohort in the remaining experimental groups (* $P < 0.05$). (b) Data are representative of one experiment with at least three mice per cohort (* $P < 0.01$ for PBS vs. C5aR antagonist and PBS vs. the combined treatment). (c) Data are representative of four independent experiments with average five mice per cohort in each; * $P < 0.01$ and * $P < 0.05$ for PBS vs. C5aR antagonist and PBS vs. the combined treatment, respectively; two-way ANOVA for all the graphs.

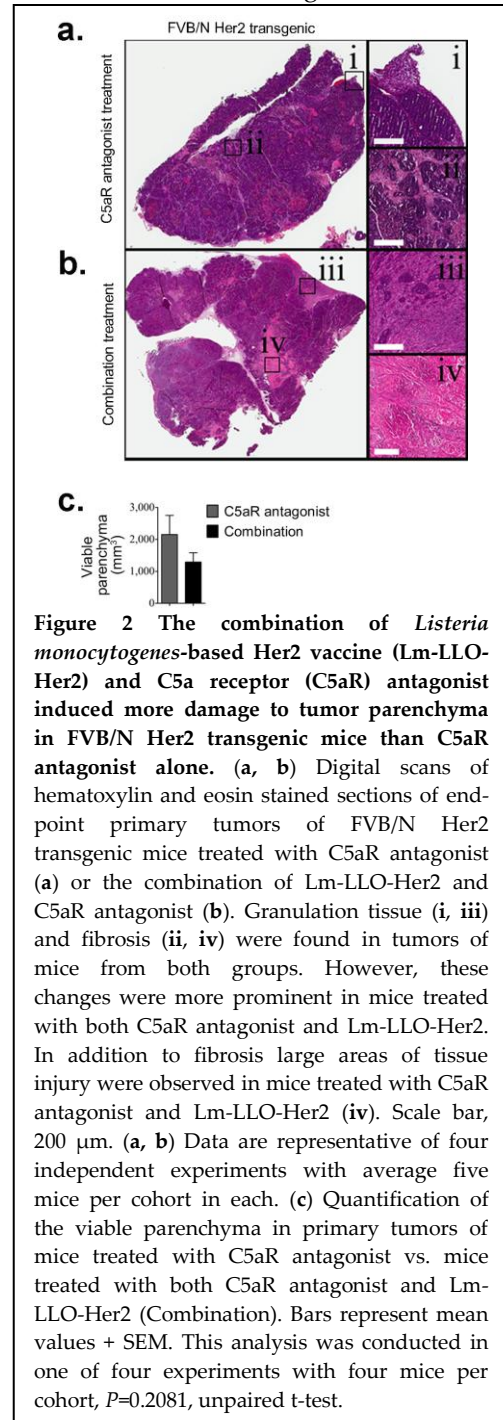
administrated intraperitoneally (i.p.) every week, since this regimen was found to be optimal in reducing tumor growth².

Therapeutic efficacy of C5aR inhibition, Her2-*Listeria monocytogenes*-delivered vaccine (Lm-LLO-Her2), and the combination of both treatments was tested in six-to-eight weeks-old female FVB/N wild-type mice inoculated s.c. with 1×10^6 (NT-2 or NT-5) tumor cells on the right flank on day 0. On day 7 after tumor cell inoculation, mice were injected with 10^7 c.f.u. of Lm-LLO-Her2 i.p. Subsequently, the complement inhibitor was administrated at every alternate day and this treatment continued until mice were sacrificed. The vaccine administration was repeated two more times on day 14 and 21 after the first dose. We observed that either Lm-LLO-Her2 or C5aR inhibitor alone reduced tumor growth in FVB/N mice bearing NT-2 tumors (Fig. 1a). Importantly, combining C5aR inhibition with Lm-LLO-Her2 further improved the efficacy of this vaccine in reducing tumor growth (Fig. 1a). C5aR inhibitor was also efficient in reducing the growth of NT-5 tumors (Fig. 1b). Lm-LLO-Her2 appeared to be less effective in this experimental setting. However, adding C5aR inhibitor to vaccine improved the efficacy of Lm-LLO-Her2 in reducing tumor growth (Fig. 1b).

Similar experiments were performed in FVB/N Her2 transgenic mice bearing NT-5 tumors. In this model mice are tolerant to antigens expressed by tumor cells and, therefore, tumors are particularly difficult to cure by immunotherapy². We found that either treatment with C5aR inhibitor alone or in combination with Lm-LLO-Her2 reduced tumor growth to similar extent. Importantly, both of these treatment regimens that included C5aR blockade were found more effective than Lm-LLO-Her2 alone (Fig. 1c).

(ii) The inhibition of C5aR combined with Lm-LLO-Her2 accelerates tumor cell death.

Even though tumor volumes in mice receiving C5aR inhibitor and mice treated with the combination of this inhibitor and Lm-LLO-Her2 were not different at the end point of studies (Fig. 1c), histology analysis revealed that the viable tumor tissue in mice treated with the combination of C5aR inhibitor and Lm-LLO-Her2 was often replaced by necrosis, fibrosis and granulation tissue (Fig. 2b). These histological features of tumor regression were also present in mice treated only with C5aR inhibitor (Fig. 2a); however, the extent of these changes was greater in mice receiving the combination therapy. Histological features of tumor regression were associated with the reduced volume of viable tumor parenchyma in mice treated with the combination therapy, as demonstrated by image analysis algorithms applied to digital slides obtained through scanning histological sections (Fig. 2a, b, c). Thus, we concluded that the combination of C5aR blockade and Lm-LLO-Her2 was superior to Lm-LLO-Her2 alone or C5aR blockade alone in reducing tumor growth. Importantly,

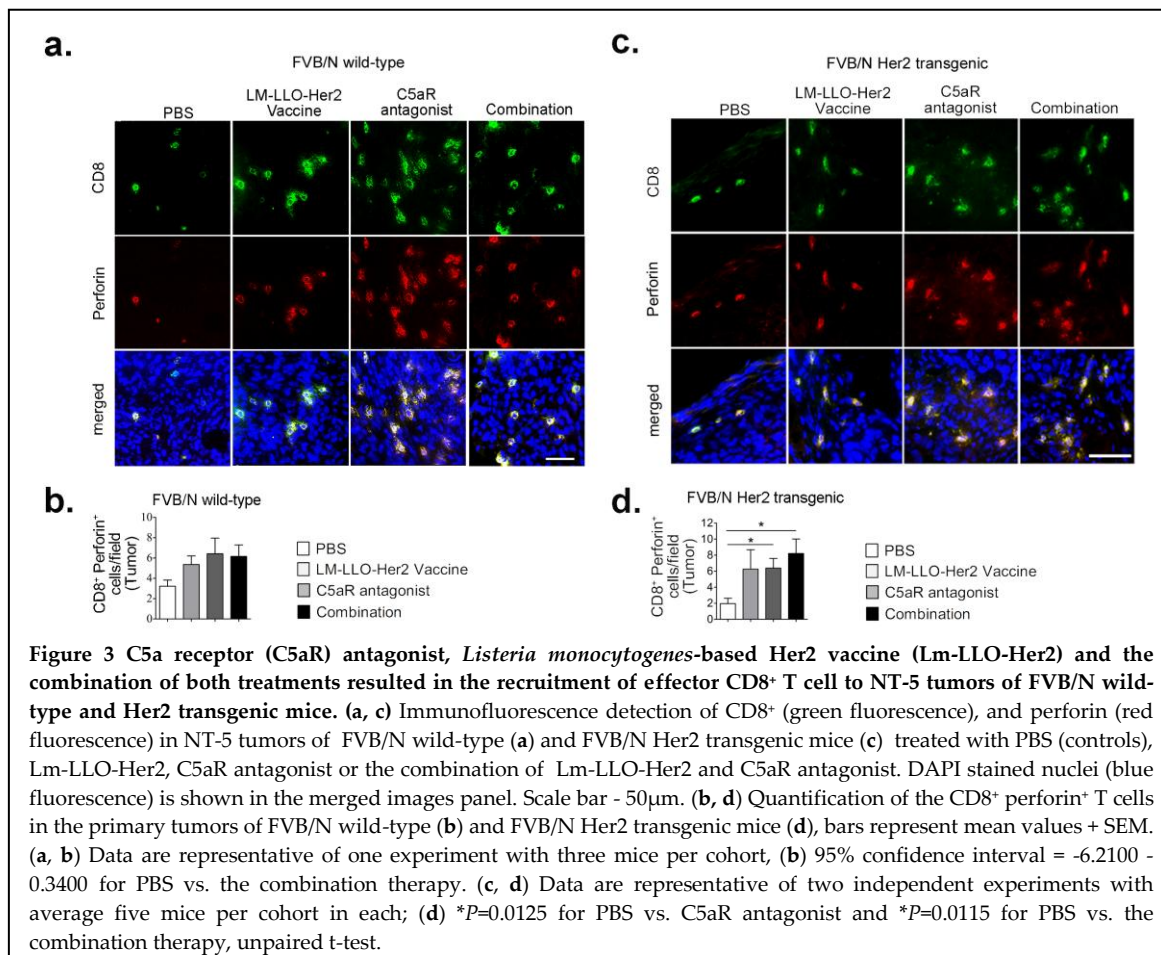


histological features of tumors from mice administered with Lm-LLO-Her2 and C5aR inhibitor suggest that tumor cells were eliminated early enough in the course of this therapy to allow for a healing process to proceed to the advanced stages, during which the mature granulation tissue and fibrosis were observed.

Aim 3 (TASK 2: months 1-6 and TASK 3: months 6-12)

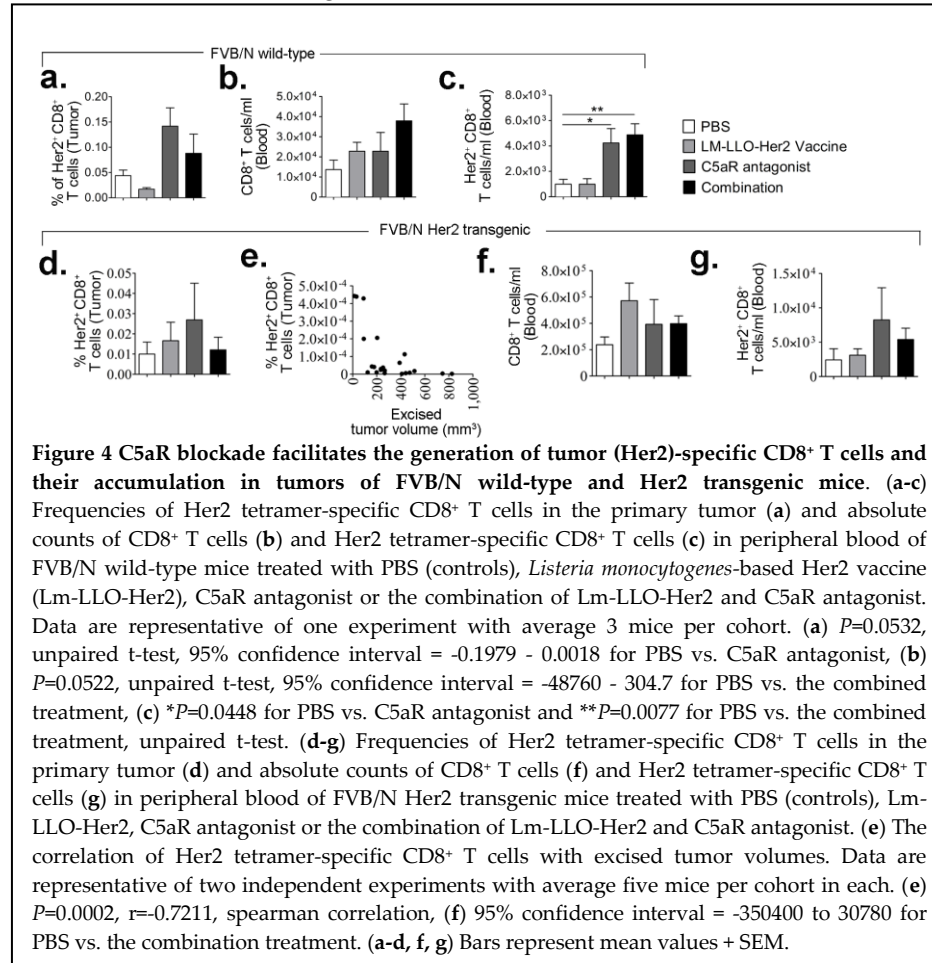
(i) Tumor cell killing triggered by therapeutic interventions involving C5aR blockade and Lm-LLO-Her2 is mediated by effector CD8⁺ T cells.

Given the pivotal role of CD8⁺ T cells in eliminating tumor cells, we examined whether the observed reduction in tumor growth/volumes after various treatments is associated with an increased infiltration of tumors by CD8⁺ T cells. These studies were performed in both the experimental systems (FVB/N wild-type and FVB/N Her2 transgenic mice). We found that tumors from FVB/N wild-type mice treated with Lm-LLO-Her2, C5aR inhibitor or the combination of both had more perforin-armed CD8⁺ T cells in their tumors than untreated control mice that received only PBS injections (Fig. 3a, b). No significant differences were found between FVB/N wild-type mice treated with Lm-LLO-Her2, C5aR inhibitor or the combination of both (Fig. 3 a, b). Also tumors of FVB/N Her2 transgenic mice treated with C5aR inhibitor, vaccine or combination of both were more infiltrated with CD8⁺perforin⁺ T cells than tumors from control mice (Fig. 3c, d). However, in a transgenic mouse model, tumors from mice receiving both vaccine and C5aR inhibitor had slightly more CD8⁺perforin⁺ T cells than tumors from mice treated only with vaccine or C5aR inhibitor alone (Fig. 3c, d). Since tumor infiltrating CD8⁺ T cells expressed perforin indicating their activation and tumorocidal activity, we concluded that these cells are actively engaged in treatment-induced anti-tumor T cell responses. Therefore, it is likely that mechanisms responsible for reducing tumor growth by various forms of tested treatments involve the mobilization of effector CD8⁺ T cells to tumors.



(ii) C5aR blockade facilitates the generation of tumor (Her2)-specific CD8⁺ T cells and their accumulation in the tumors.

Given the C5aR blockade enhanced CD8⁺ T cell infiltration of tumors in FVB/N wild-type and Her2 transgenic mice (Fig. 3), we postulate that C5aR signaling impairs the recruitment of anti-tumor CD8⁺ T cells to tumors. To test this hypothesis, single cell preparations from tumors and blood of NT-5 tumor-bearing FVB/N wild-type and Her2 transgenic control mice and mice treated with C5aR inhibitor, Lm-LLO-Her2 or combination of both agents were prepared. Next, these cells were stained with CD8a antibody and Her2-tetramer and analyzed by flow cytometry. Higher frequencies of tumor/Her2-specific CD8⁺ T cells were observed in tumors of mice that received C5aR inhibitor when compared to control mice or mice treated only with Lm-LLO-Her2 (Fig. 4a, d). In FVB/N wild-type mice, adding C5aR inhibitor to Lm-LLO-Her2 increased frequencies of tumor/Her2 specific T cells in tumors in comparison to Lm-LLO-Her2 alone (Fig. 4a), however, such effect has not been observed in transgenic mice (Fig. 4d).



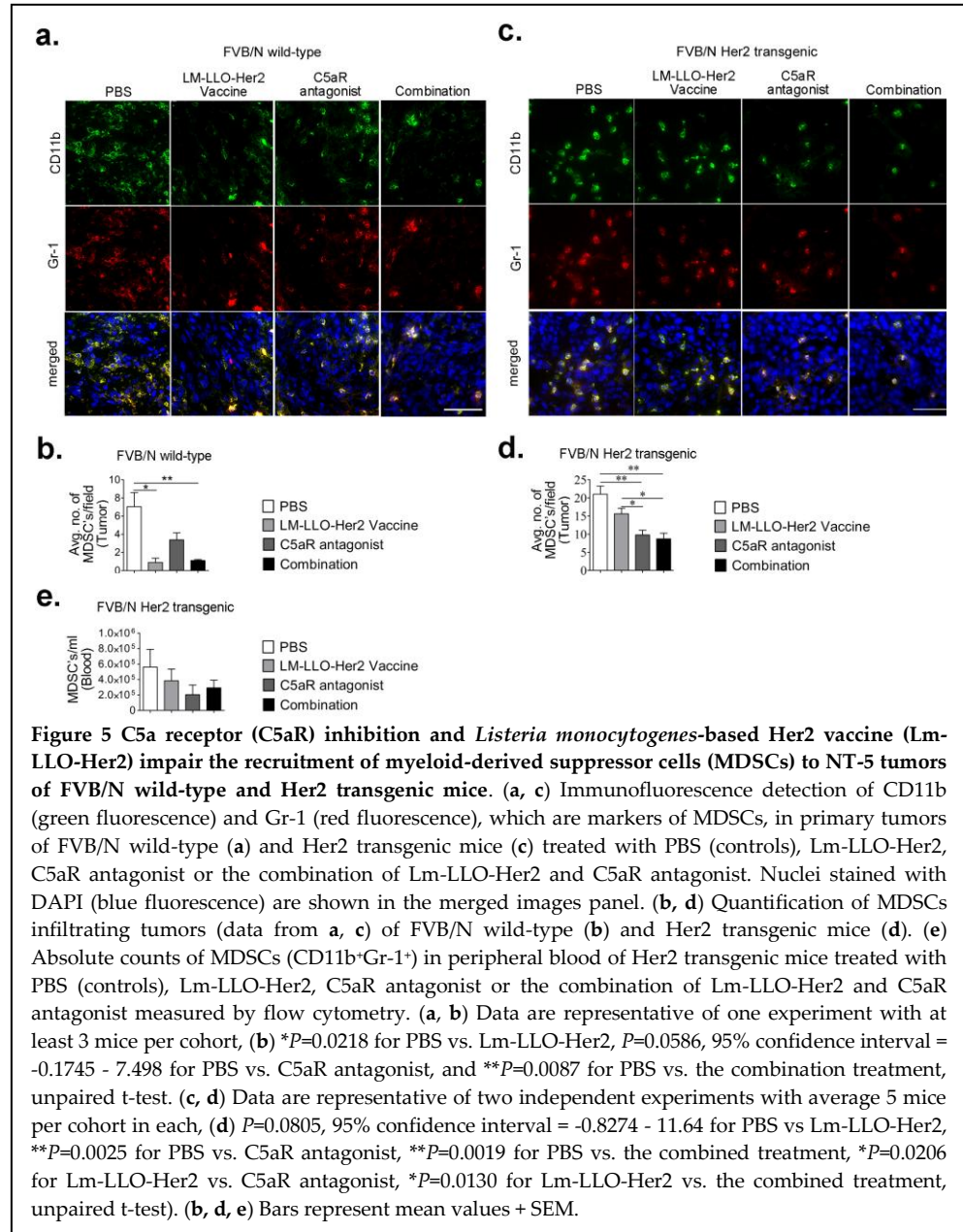
in transgenic mice, Lm-LLO-Her2 alone induced the highest increase in circulating CD8⁺ T cells (Fig. 4f). Tumor-specific/Her2 CD8⁺ T cells were induced in peripheral blood by C5aR inhibitor and the combination of this inhibitor with vaccine (Fig. 4c, g). However, Lm-LLO-Her2 alone was not efficient in inducing tumor-specific/Her2 CD8⁺ T cells at the periphery in both mouse strains (Fig. 4c, g).

(iii) The inhibition of C5aR signaling in combination with Lm-LLO-Her2 attenuated immunosuppression in the tumor microenvironment by blocking the recruitment of myeloid-derived suppressor cells (MDSCs) to tumors.

The impact of tumor-specific/Her2 T cells on tumor growth is underscored by the inverse correlation between tumor volumes and frequencies of tumor/Her2-specific T cells in tumors of transgenic mice (Fig. 4e).

Various treatments also increased total numbers of CD8⁺ T cells in peripheral blood of both FVB/N wild-type and transgenic mice (Fig. 4b, f). However, in wild-type mice, the most prominent increase in circulating CD8⁺ T cells was observed in mice treated with both C5aR inhibitor and Lm-LLO-Her2 (Fig. 4b), whereas

Since myeloid-derived suppressor cells (MDSCs) are important immunosuppressive cell population recruited by tumors^{3, 4} and our previous studies suggested that C5a activates and recruits these cells to tumors⁵, we investigated the impact of various treatments on the MDSC recruitment to tumors. We hypothesize that disabling MDSCs is one of mechanisms by which C5aR blockade can improve the efficacy of anti-tumor vaccination. We found that all treatments reduced tumor infiltrating MDSCs when compared to control mice in both FVB/N wild-type and FVB/N Her2 transgenic mice-bearing NT-5 tumors (Fig. 4a, b, c, d). Interestingly, Lm-LLO-Her2 was more efficient in reducing tumor-infiltrating MDSCs in FVB/N wild-type mice than in transgenic mice. In addition, Lm-LLO-Her2 alone caused a slight decrease in circulating MDSCs in transgenic mice bearing NT-5 tumors, whereas C5aR inhibition reduced MDSCs in peripheral blood to a greater extent than the vaccine alone or the combined treatment (Fig. 5e). Thus, it appears that C5a regulates MDSC infiltration of tumors through the recruitment of these cells from the circulation and also by affecting quantities of circulating MDSCs. Although Lm-LLO-Her2 had similar



effects on this immunosuppressive cell population in tumors, the reduction of immunosuppression by vaccine seems to be less apparent than in the case of C5aR blockade. Therefore, we concluded that the combination of Lm-LLO-Her2 with C5aR blockade is the most efficient in reducing malignancy-associated immunosuppression.

Key research accomplishments:

- C5a receptor (C5aR) blockade reduced tumor growth in several syngeneic and transgenic models of breast cancer.
- The extent of this reduction was comparable to or better than the therapeutic effect of *Listeria monocytogenes*-delivered Her2 vaccine (Lm-LLO-Her2).
- C5aR inhibition synergized with Lm-LLO-Her2 in limiting tumor growth.
- These therapeutic effects were associated with the enhanced recruitment of tumor-specific CD8⁺ T cells to tumors.
- C5aR inhibition alone contributed to this recruitment and induced tumor-specific T cell responses at the periphery.
- Mechanisms of enhanced anti-tumor responses after various treatments were associated with the attenuation of tumor-induced immunosuppression, since C5aR blockade reduced numbers of highly immunosuppressive myeloid-derived suppressor cells in tumors of Her2 transgenic mice.

Reportable outcomes:

- *Manuscripts, abstracts, presentations:* “Antitumor activity of a monoclonal antibody targeting major histocompatibility complex class I-Her2 peptide complexes”. Jain R, Rawat A, Verma B, Markiewski MM, Weidanz JA. *J Natl Cancer Inst.* 2013 Feb 6;105(3):202-18. doi: 10.1093/jnci/djs521. Epub 2013 Jan 8
- *Funding applied for based on work supported by this award:* NIH grant application entitled Crosstalk between stem cells and innate immunity in the pre-metastatic niche; R01 CA181061-01, PI: Maciej Markiewski.

Conclusions:

We have determined in syngeneic and transgenic models of Her2-expressing breast cancer that the complement anaphylatoxin C5a receptor (C5aR) blockade is efficient immunotherapy for this malignancy either in a form of monotherapy or in a combination with *Listeria monocytogenes*-based Her2 vaccine (Lm-LLO-Her2). Importantly, C5aR inhibition synergized with Lm-LLO-Her2 in limiting tumor growth. Mechanisms of therapeutic response to C5aR inhibition were related to the attenuation of tumor-induced immunosuppression and the subsequent initiation of anti-tumor T cell responses.

References:

1. Reilly RT, Gottlieb MB, Ercolini AM, Machiels JP, Kane CE, Okoye FI, *et al.* HER-2/neu is a tumor rejection target in tolerized HER-2/neu transgenic mice. *Cancer research* 2000, **60**(13): 3569-3576.

2. Singh R, Paterson Y. In the FVB/N HER-2/neu transgenic mouse both peripheral and central tolerance limit the immune response targeting HER-2/neu induced by *Listeria monocytogenes*-based vaccines. *Cancer immunology, immunotherapy : CII* 2007, **56**(6): 927-938.
3. Ostrand-Rosenberg S, Sinha P. Myeloid-derived suppressor cells: linking inflammation and cancer. *Journal of immunology* 2009, **182**(8): 4499-4506.
4. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nature reviews Immunology* 2009, **9**(3): 162-174.
5. Markiewski MM, DeAngelis RA, Benencia F, Ricklin-Lichtsteiner SK, Koutoulaki A, Gerard C, *et al.* Modulation of the antitumor immune response by complement. *Nature immunology* 2008, **9**(11): 1225-1235.